Article title: Investigation on the inhibitory effects of Zingiber officinale (ginger) and Curcuma longa (turmeric)’s antifungal properties on Rhizopus stolonifer (black bread mould) growth on bread

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Preprint statement: This article is a preprint and has not been peer-reviewed, under consideration and submitted to
ScienceOpen Preprints for open peer review.

DOI: 10.14293/S2199-1006.1.SOR-.PPADJ9P.v1

Preprint first posted online: 30 October 2022

Keywords: Zingiber officinale, Curcuma longa, Rhizopus stolonifer, Antifungal Properties, Fungi Growth
Investigation on the inhibitory effects of *Zingiber officinale* (ginger) and *Curcuma longa* (turmeric)’s antifungal properties on *Rhizopus stolonifer* (black bread mould) growth on bread

by B. K.
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Introduction

The scope of this internal assessment aims to explore effective ways to prevent the bread from moulding for as long as possible along with the herbaceous plants with fungi inhibiting properties. This topic was chosen as mould growth in food substances is a problem I occasionally encounter and have a personal engagement with in my daily life. Mould begins to cover products such as bread, fruit, and cheese that were not consumed within their expiration date. In such cases, we may have to throw away this food item as a waste. I aim to propose a solution to an aspect of this problem by conducting an experiment on the growth of Rhizopus stolonifer which is commonly known as black bread mould. In this experience, I aim to demonstrate how growth rates of moulds, which belong to the fungi kingdom, will change against substances with antifungal properties by testing them on Rhizopus stolonifer. Therefore, I have chosen to use the two herbs Z. officinale and C. longa and in this investigation, I examined their inhibitory activity on fungi as well as comparing which of these two herbs is more effective in inhibiting fungal growth and reproduction.

The essay investigates the growth of the fungus Rhizopus stolonifer after the Zingiber officinale and Curcuma longa treatment. The research is relevant as it brings new perspectives to the biological aspects of a real life problem that most humans encounter in their daily lives. In order to investigate the inhibitory effects of the herbaceous plants with antifungal properties, I conducted an experiment with the herb species Zingiber officinale and Curcuma longa. For this purpose, I compared the experimental groups under the same circumstances, with an independent variable of two types of antifungal herbaceous plant treatments alongside a control group that shares the same conditions except for the herbaceous plant treatment. For the antifungal treatment, Z. officinale and C. longa herbs were directly applied inside the pieces of bread.

Research Question

In my internal assessment, I will be answering the research question ‘To what extent Zingiber officinale (ginger) and Curcuma longa (turmeric) inhibit the growth of Rhizopus stolonifer (black bread mould) on bread?’

Background Information

Zingiber officinale

Zingiber officinale, which is commonly known as ginger, is an herbaceous plant that belongs to the Zingiberaceae family. These plant species are usually distributed in tropical areas. The plants that display antifungal properties consist of a wide range of various vitamins and minerals such as Vitamin C, iron, magnesium, calcium, potassium, and sodium. It has many commercial usage areas
from medicine to culinary. It is an herb used for medicinal purposes as well as being used as a flavouring spice in dishes all over the world. 

Curcuma longa

Curcuma longa, which otherwise goes by the name of turmeric, is an herbaceous plant belonging to the Zingiberaceae family similar to the Z. officinale. The plant species, that show antifungal properties, is known to originate from the South Asia region. C. longa, which is rich in vitamins A, B, and C, also has antioxidant properties in terms of abundant phenolic and flavonoid amounts. Turmeric, which is also widely used in dishes all around the world, has yellow flowers and it gives its yellow color to the dishes it is used in. This feature of the C. longa also allowed their experimental groups to be easily distinguished in this experiment.

Rhizopus stolonifer

Rhizopus stolonifer, known by the name black bread mould, is one of the most common fungi species on earth. The species that belong to the genus Rhizopus, is a saprotroph species that spreads rapidly through stolons and usually grows organic matter such as food like bread, bananas, and grapes. Rhizopus stolonifer which can reproduce sexually in favorable circumstances, in most cases reproduces asexually and form a colony of spores. The structure of the Rhizopus stolonifer spores consists of the parts sporangium, spores, sporangiophore, stolon, and rhizoids as illustrated in Figure 2.

Antifungal Properties

Antifungal properties are properties that inhibit the growth and reproduction of organisms belonging to the kingdom of fungi. There are natural antifungal agents as well as artificial antifungals produced by the chemical industry. Zingiber officinale and Curcuma longa are two examples of natural agents that can demonstrate antifungal capacities.

Preliminary Testing

The preliminary testing was performed in order to create the ideal conditions for the experimental environment and to check whether the dependent variables provide results that would

allow the investigation to attain a significant impact. Controlled variables and the conditions for the storage such as the amount of sunlight that the breads will be exposed to were chosen accordingly based on the findings from the preliminary testing. The preliminary testing revealed that 25°C Celsius with no direct sunlight provides the ideal conditions for the mould growth. In this way, the most suitable conditions for the observation of the growth of fungi and the antifungal properties of Z. officinale and C. longa were created. The preliminary testing also revealed that ginger and turmeric were the most suitable options among the antifungal herbs for this experiment. These two plants were chosen because they would provide a strong argument for investigating the aim of the experiment.

Hypotheses & Explanation

Null Hypothesis (H₀): If Zingiber officinale and Curcuma longa are not effective in inhibiting the growth of Rhizopus stolonifer, then the mould growth will occur during the same time period with the control group.

Alternate Hypothesis (Hₐ): If Zingiber officinale and Curcuma longa inhibit the growth of Rhizopus stolonifer for a longer time, then the mould growth will occur in the experimental groups with Z. officinale and C. longa later than the control group.

Justification: If the Zingiber officinale and Curcuma longa have antifungal properties and Rhizopus stolonifer (black bread mould) is produced by the fungi then the two Zingiberaceae species will make the bread more resistant to the moulding by preventing the rapid growth of Rhizopus stolonifer on breads.

Variables

Independent Variables

1) The types of antifungal Zingiberaceae species: Z. officinale and C. longa - The independent variables were manipulated while setting up the experimental groups. Ginger and turmeric were added to the different experimental groups during the preparation of the breads. Each was used in 10±0.1 milligram units.

Dependent Variables

1) The growth rate of Rhizopus stolonifer - The growth rate was measured by observing the changes in the presence of the Rhizopus stolonifer each day. Estimations were made in terms of mould growth per day.

2) The growth zone of Rhizopus stolonifer - The area where the moulding spread was measured with a grit as explained in the Methodology. The measurements were calculated in cm measurement units.
## Controlled Variables

<table>
<thead>
<tr>
<th>Controlled Variables</th>
<th>Justification for controlling</th>
<th>How method allows for the control of these variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>The temperature at which the breads are stored</td>
<td>The moulding rate may vary at different temperatures. Therefore it needs to be controlled.</td>
<td>The room that the breads are located in has a maintained temperature of 25°C. It is ideal to use the room temperature value for the experiment to bring a new tangible approach to the daily life situation.</td>
</tr>
<tr>
<td>Preparation of breads</td>
<td>Different ingredients inside the bread or the same ingredients with different proportions may show features that could potentially alter the results.</td>
<td>The same steps should be followed for the preparation of all breads. Furthermore, the identical ingredients should be used in the same proportions, except for the type of antifungal Zingiberaceae species which is the independent variable.</td>
</tr>
<tr>
<td>The amount of sunlight the breads receive</td>
<td>The presence of sunlight impacts the fungal activity of the <em>Rhizopus stolonifer</em>.</td>
<td>The experiment was carried out in a dark room, as too much sunlight would affect the mould growth.</td>
</tr>
<tr>
<td>The sterile environment conditions which the breads are stored in</td>
<td>The moulding rate may be influenced by the interactions of an external substance with bread. Furthermore, the reproduction rate of fungi changes in contact with the air.</td>
<td>The experiment was conducted in the school laboratory to provide a sterile and hygienic environment for the experimental groups. The breads were stored in sealed bags to prevent air and substance exposure.</td>
</tr>
<tr>
<td>The time intervals in which the moulds are examined</td>
<td>In order for the experiment to give reliable results, it is necessary to examine the experimental groups using the same methods. Observations made at different time intervals would impair the accuracy of the experiment.</td>
<td>The experiment results were regularly observed and noted every day in the mornings.</td>
</tr>
</tbody>
</table>

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Materials And Method

Materials/Apparatus

Microscope
Forceps
Micropipette (±0.1 mL)
Coverslips
Glass slides
15±0.1 mL Methylene blue aq solution
A metric ruler (±0.05 cm)
A pair of gloves
A pair of safety goggles
A lab coat
Filter paper
A transparent gridded sheet
15 Plastic ziplock bags
50±0.5 grams of powder turmeric
50±0.5 grams of powder ginger
35±0.5 grams of instant yeast
3400±0.5 grams of flour
20±0.5 grams of granulated sugar
75±0.5 grams of salt
1750±1.0 milliliters of warm distilled water
150±1.0 milliliters of olive oil

Methodology

1) Prepare the breads and set up the experiment
   A) Prepare one plain bread.
   B) Prepare one bread adding 10 grams of Curcuma longa.
   C) Prepare one bread adding 10 grams of Zingiber officinale.
   D) Moisten the breads for the moulding to be observed clearly and in a short time span.
   E) Put the breads in plastic ziplock bags and seal them so that they will not be exposed to air.
   F) Label the storage bags according to their type of antifungal Zingiberaceae treatment.

2) Observe the moulding
   A) Store the breads at 25° Celsius room temperature in a dark room.
   B) Wait for a day for the results to come out.
   C) Record and take note of your findings and observations results.
   D) Repeat steps B and C every day for 10 days.

3) Investigation under microscope
   A) Take samples of the Rhizopus stolonifer from the plain bread with the help of a forceps while wearing gloves.
   B) Place the Rhizopus stolonifer samples collected from the first bread on the glass slide.
C) Transfer 1±0.1 mL methylene blue aq solution on the glass side by using micropipettes.
D) Cover the top of the glass slide with a coverslip.
E) Remove the excess solution from the edge of the slide with the help of filter paper.
F) Set up the microscope and place the slide inside.
G) Observe the mould under 40X, 100X, and 400X total magnifications, as shown in Table 1, with the help of a light microscope.
H) Continue the steps A-G for the experimental groups of *Z. officinale* and *C. longa.*

<table>
<thead>
<tr>
<th>Objective Lens Powers</th>
<th>Power of the Eyepiece</th>
<th>Total Magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X</td>
<td>10X</td>
<td>4X.10X= 40X</td>
</tr>
<tr>
<td>10X</td>
<td>10X</td>
<td>10X.10X= 100X</td>
</tr>
<tr>
<td>40X</td>
<td>10X</td>
<td>40X.10X= 400X</td>
</tr>
</tbody>
</table>

4) Calculate the area of the moulding zone
   A) Overlay the transparent gridded sheet on the photographs of the breads so that the mould is not damaged for the following measurements.
   B) Count the number of squares covered by mould and record the findings.

The procedure was repeated the same way for 5 trials for each of the experimental groups.

**Safety considerations and risk assessment**

**Safety and environmental concerns** - Necessary precautions were taken when handling the *Rhizopus stolonifer.* In order not to cause any respiratory health problems, the mould should not be inhaled for a long time. For this purpose, the allocated time spent with *Rhizopus stolonifer* was limited. There was no direct contact or exposure to the mould. Necessary protective equipment and clothing such as gloves, a lab coat, and goggles were utilized at all times throughout the experiment. When necessary, the breads were transferred carefully with the help of the appropriate apparatus. Furthermore, frequent hand washing was emphasized after every observation of the moulding and breads were disposed of after the final observation.

**Ethical concerns** - There were no prominent ethical issues in this experiment.

**COVID-19** - As the experiment was conducted during the COVID-19 pandemic, strict safety regulations were followed and extra care was taken such as social distancing and wearing masks all throughout the experiment.
Results

Raw Data

Images given below in Figure 3 and Figure 4 are sample photographs of the experiment results that I took with a phone. Black bread moulds were examined under the microscope and sporangium, spores, sporangiophore structures of the mould were observed.

**Figure 3:** Sample photograph of the *Rhizopus stolonifer* collected from bread surface

**Figure 4:** Sample microscope image of *Rhizopus stolonifer* collected from bread surface (100X)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Time (days)</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
<th>Trial 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>After <em>Z. officinale</em> treatment</td>
<td></td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>After <em>C. longa</em> treatment</td>
<td></td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Control Group</td>
<td></td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>The final area of the moulding zone (cm²) (±1.0)</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
<th>Trial 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>After <em>Z. officinale</em> treatment</td>
<td></td>
<td>38.43</td>
<td>36.54</td>
<td>34.22</td>
<td>36.48</td>
<td>39.68</td>
</tr>
<tr>
<td>After <em>C. longa</em> treatment</td>
<td></td>
<td>26.56</td>
<td>24.44</td>
<td>22.50</td>
<td>21.93</td>
<td>28.62</td>
</tr>
<tr>
<td>Control Group</td>
<td></td>
<td>122.08</td>
<td>112.35</td>
<td>119.84</td>
<td>114.66</td>
<td>132.16</td>
</tr>
</tbody>
</table>
In Table 4, a summary of the data obtained from the experiment was demonstrated and the sum, mean, and standard deviations of each experimental group were calculated. The mean calculations were made using the following formula:

\[
\bar{x} = \frac{\sum x_i}{n} = \frac{x_1 + x_2 + \ldots + x_n}{n}
\]

Standard deviation was calculated with the following formula:

\[
\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}
\]

where \(\bar{x}\) is the population mean, \(\sigma\) is the standard deviation, and \(n\) is the total size of the population. An example of the calculations is given below:

\[
\bar{x} = \frac{38.43 + 36.54 + 34.22 + 36.48 + 39.68}{5} = 37.07 \pm 1.0 \text{ is the mean area of mould growth after } Z. \text{ officinale treatment}
\]

\[
\sum (x - \bar{x})^2 = 17.4132
\]

\[
\sigma = \sqrt{\frac{17.4132}{4}} = \sqrt{4.3533} = 2.0865 \text{ is the standard deviation after } Z. \text{ officinale treatment}
\]
In Table 5, the percentage of the mean area that moulds covered over the bread’s total area was calculated. It can be inferred from this table that there is a significant difference between the data. To check and confirm this mean difference between the datasets, the ANOVA test will be applied further in this investigation.

Table 5: Processed data of moulded area percentage

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Percentage of Mean Moulded Area over the Total Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>After <em>Z. officinale</em> treatment</td>
<td>( \frac{37.07}{132.25} \times 100 = 28.03 % )</td>
</tr>
<tr>
<td>After <em>C. longa</em> treatment</td>
<td>( \frac{24.81}{132.25} \times 100 = 18.76 % )</td>
</tr>
<tr>
<td>Control Group</td>
<td>( \frac{120.218}{132.25} \times 100 = 90.90 % )</td>
</tr>
</tbody>
</table>

### Statistical Data

**ANOVA**

In this experiment, one way ANOVA Test, also known as the Analysis of Variance Test, was performed to determine whether *Z. officinale* and *C. longa* influence the growth rate of mould. The reason for this statistical test was in order to check if there is a statistical difference between the three data groups and determine the impact of different types of antifungal Zingiberaceae species on the inhibiting of the *Rhizopus stolonifer*. The following hypotheses were constructed for the ANOVA Test.

**Null Hypothesis** (H₀) : There is no statistically significant difference between the mean final areas of mould growth of *Zingiber officinale*, *Curcuma longa*, and the control group at the end of the 10th day.

**Alternate Hypothesis** (Hₐ) : There is a statistically significant difference between the mean final areas of mould growth of *Zingiber officinale*, *Curcuma longa*, and the control group at the end of the 10th day.

Table 6: ANOVA Test results

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Square (SS)</th>
<th>Degree of Freedom (df)</th>
<th>Mean of Square (MS)</th>
<th>Variation (F)</th>
<th>Probability Level (P-value)</th>
<th>F-critical value (at 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>26944.3066</td>
<td>2</td>
<td>13472.1533</td>
<td>561.77416</td>
<td>&lt;.00001</td>
<td>3.8853</td>
</tr>
<tr>
<td>Within Groups</td>
<td>287.7773</td>
<td>12</td>
<td>23.9814</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27232.0839</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The ANOVA results shown in Table 6 were calculated with an Excel sheet. Whether to accept the null hypothesis depends on the comparison of the F with F-critical. If the F is greater than the F-critical value, it indicates that there is a significant difference between the means of the samples and therefore, the null hypothesis is rejected. In this ANOVA test, F is calculated as 561.77416 and F-critic is calculated as 3.8853 which corresponds to the degrees of freedom between groups, degrees of freedom within groups, and significance level, where p=0.05.

As the F = 561.77416 > 3.8853, H₀ is rejected and Hₐ is accepted. Therefore, it is accepted that there is a statistically significant difference between the final areas of mould growth of *Zingiber officinale*, *Curcuma longa*, and the control group at the end of the 10th day.

**Analysis & Discussion**

**Graph 1:** Bar chart demonstrating the final areas of the mould growth on the 10th day (with the uncertainty ±1.0)

From all the data values obtained from this experiment and from the bar graph plotted above labelled as Graph 1, it can be interpreted that *Zingiber officinale* and *Curcuma longa* both inhibit the growth of *Rhizopus stolonifer* mould. The fastest mould growth is evident in the control group without any antifungals. Moulds in the control group, which started to be observed in an average of 3.2 days, were also the group that covered the largest portion of bread in terms of area with a 90.9% ratio. Following that, the *Zingiber officinale* treatment experimental group started to display moulding in 5.2 days and covered 28.03% of the bread by the end of the 10 days. There is a 62.87 percent difference between these two ratios of the control group and *Zingiber officinale* treatment groups which clearly indicates that the *Z. officinale* is an effective inhibitor of fungi and especially *Rhizopus stolonifer* reproduction. Meanwhile, the mean of the first moulding presence in the *Curcuma longa* experimental group occurs on the 6th day. At the end of the experiment, on the 10th day, the *C. longa* treatment group covered only 18.76% of the bread. It can be deduced that the experimental group, whose mean moulded area over the total bread area was 72.14 percentile less than the control group.

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Conclusion & Evaluation

Conclusion

In conclusion, the research question ‘To what extent Zingiber officinale (ginger) and Curcuma longa (turmeric) inhibit the growth of Rhizopus stolonifer (black bread mould) on bread?’ was explored in this investigation. The internal assessment’s aim was to investigate how the independent variables of two different types of Zingiberaceae species affect the dependent variables, growth rate and growth zone of Rhizopus stolonifer. For this reason, the inhibitory effects of the two herbaceous Zingiberaceae plants with antifungal properties, Zingiber officinale (ginger) and Curcuma longa (turmeric), were examined by conducting an experiment. The qualitative and the quantitative data collected from the experiment supported the alternative hypothesis which suggests that Z. officinale and C. longa will inhibit the growth of Rhizopus stolonifer for a longer time and therefore the mould growth will occur in the experimental groups with Z. officinale and C. longa later than the control group. The scientific reasoning behind the two Zingiberaceae species making the bread more resistant to mould is their antifungal properties which inhibit the rapid growth of Rhizopus stolonifer mould, which is produced by fungi. As the data from Table 5 reveals the Z. officinale and C. longa treatment groups demonstrated lower mould growth rate trends compared with the control group. Between these treatment groups, it can be deduced that C. longa is more effective in inhibiting the growth of fungi and the spreading of mould than Z. officinale. The further processed data by performing ANOVA statistical test also accepted the alternative hypothesis and revealed that there is a statistically significant difference between the mean areas of mould growth zones of Zingiber officinale, Curcuma longa, and the control group at the end of the 10th day.

Evaluation

Strengths - The investigation was successful as the experiment fulfilled its targeted objectives and provided supporting evidence to the hypothesis. Preliminary testing was carried out before the actual experiment in order to provide the most suitable conditions for the mould to be observed and recorded. After 5 experiment trials conducted according to the findings obtained from the preliminary testing results, it was possible to achieve accurate and reliable results with low uncertainties. The results obtained from the experiment were then processed and statistical tests were used which allowed me to understand the relationship between the independent and dependent variables. This exploration helped me to develop new approaches towards biological concepts while investigating a situation I encounter in my life. In addition, no accidents occurred during the experiment due to the strict adherence to safety regulations.

Weaknesses and Limitations - Similar to many research, this investigation also has its weaknesses and limitations. For instance, even though the breads were kept in airtight storage bags, they were taken out of the bag in order to observe and measure the mould growth rate. During this period, the breads contacted with the air which may slightly alter the results. This constitutes a weak point of the experiment. Furthermore, limited access to analytical laboratories under the COVID-19 regulations restricted the further advanced measurements and calculations to support the experiment.
In order to improve these weaknesses and limitations, I suggest repeating the experiment in an analytical laboratory at a time when the safety regulations allow the experiment to be performed.

**Further Extensions** - To further explore the topic of the fungi inhibitory effects of the *Z. officinale* and *C. longa*, whether the two herb species inhibit the growth of other fungal species can be investigated in future research. The effectiveness of different antifungal Zingiberaceae plant species on inhibition of the *Rhizopus stolonifer* growth and reproduction would also be a good extension that can contribute to this research.

**Bibliography**


